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Measuring cytokine levels in blood

Importance of anticoagulants, processing, and storage conditions

P.W. Thavasu [a], S. Longhurst [a], S.P. Joel [a], M.L. Slevin [a] and F.R. Balkwill [b]

[a] Imperial Cancer Research Fund, Department of Medical Oncology, Homerton Hospital, London E9 6SR, UK, and [b] Imperial Cancer Research Fund, Biological Therapies Laboratory, Lincoln's Inn Fields, London WC2A 3PX, UK

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The stability and recovery of six human recombinant cytokines (tumour necrosis factor (TNF), interferon-α (IFN-α), IFN-γ, interleukin-1α (IL-1α), IL-1β, and IL-6) from whole blood was investigated with a view to optimizing blood collection and storage procedures prior to performing immunoassays. Blood from healthy volunteers was subjected to various processing and storage procedures. Blood samples were treated with either: ethylenediamine tetraacetic acid (EDTA) (1.5 mg/ml blood) (E); EDTA/Trasylol (1.5 mg and 1000 KIU/ml blood) (ET); heparin (30 IU/ml) (H) or allowed to clot (serum). The bloods were spiked with individual cytokines, split into aliquots and kept at 4°C or RT.

In the first instance spiked bloods from healthy volunteers ($n = 5$ per cytokine) were processed using sterile and non-pyrogenic materials and procedures. At regular time intervals, samples were cold spun, separated, flash frozen and assayed for the appropriate cytokine using RIA/IRMA methods.

In a further study, timed separation was repeated with spiked blood from healthy volunteers ($n = 5$ per cytokine) using normal commercially available blood collection materials and procedures.

In a third study, spiked blood from healthy volunteers ($n = 3$ per cytokine) was processed under sterile and non-pyrogenic conditions, and the blood samples separated, aliquoted and flash frozen within half hour of collection. These were then subjected to repeated cycles of freeze thawing at 4°C or RT before assaying.

In general, the stability of cytokines in whole blood was improved by storage at 4°C and/or rapid separation. There was no significant difference between samples handled under sterile, non-pyrogenic conditions and those collected using normal blood collection procedures. The blood collection procedures described in this paper did not induce any of the six cytokines in the unspiked blood. Overall, EDTA-treated samples performed most consistently. The addition of trasylol did not significantly affect the results. Most of the cytokines appeared unaffected by up to three freeze thaw cycles. The stability and recovery of the spiked cytokines varied from least stable to most stable spiked cytokine as follows; TNF-α < IL-6 < IFN-γ < IL-1α < IFN-α < IL-1β. The recovery of spiked IFN-γ from heparinized plasma samples was considerably higher than any other plasma or serum samples. The recovery of spiked TNF-α

Correspondence to: P.W. Thavasu, Imperial Cancer Research Fund, Biological Therapies Laboratory, Lincoln's Inn Fields, London WC2A 3PX, UK.

Abbreviations: IL-1α, interleukin-1α; IL-1β, interleukin-1β; IL-6, interleukin-6; IFN-γ, interferon-γ; IFN-α, interferon-α; TNF-α, Tumour necrosis factor α; E or EDTA, ethylenediamine tetraacetic acid; ET, ethylenediamine tetraacetic acid and trasylol (aprotinin); KIU, kallikrein international units; H, heparin; S, serum; RT, room temperature; RIA, radioimmunoassay; IRMA, immunoradiometric assay; NIBSC, National Institute of Biological Standards and Control; WHO, World Health Organization.

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and IL-6 from serum samples was consistently lower than amounts recovered from plasma samples (anticoagulant treated). Thus optimal conditions for blood collection for consistent immunoassay measurements of circulating recombinant cytokines are rapid processing at 4°C of EDTA treated blood.

Key words: Blood collection; Anticoagulant; Spiked recombinant cytokine; Immunoassay; Stability; Recovery

Introduction

The increasing clinical use of cytokines in the treatment of chronic infections and malignant disease warrants a systematic approach to quantifying and assessing the immunopharmacological effects in patients receiving these agents, either as single agents or in conjunction with chemotherapy. It may also serve to explain and quantify the effects of other immune stimulants, the use of anticytokine antibodies, and possible prognostic and diagnostic use for the measurement of these cytokines.

In such studies it is important to identify optimal conditions for accurately assaying patient blood samples. Many polypeptides have short half lives in vivo and may be subjected to further degradation ex vivo (Chard, 1987). There is also the possibility of ex vivo induction of cytokine molecules during the processing of bloods (Leroux-Roels et al., 1988).

In this study we have attempted to optimize blood collection procedures and measure these polypeptide molecules in blood samples using immunological methods.

Whole bloods from healthy volunteers were spiked with individual cytokines. Six human recombinant cytokines (TNF-α, IL-6, IL-1α, IL-1β, IFN-γ and IFN-α) were assessed for their recovery and stability in serum, or plasma from bloods treated with EDTA, EDTA with Trasylol, or heparin. These samples were subjected to timed separation at 4°C and RT. Blood sampling and processing was carried out using sterile and non-pyrogenic materials and conditions, and usual normal blood collection materials and procedures. The effect of repeated cycles of freeze/thawing of plasma/serum samples at 4°C and RT prior to assaying was also studied.

Materials and methods

Cytokines

Whole blood from healthy volunteers was spiked with individual human recombinant IL-1α, IL-1β and TNF-α (WHO international reference preparations). Likewise, an interim standard preparation of human recombinant IL-6 was used to spike volunteer blood. These cytokine preparations were generously provided by Drs. S. Poole, A. Meager and R. Thorpe (National Institute of Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Potters Bar, Herts. EN6 3QG, UK). Human recombinant IFN-γ was obtained from Dr. A. Galatzka (Biogen). It was $> 99\%$ pure and had a specific activity of 2×10^7 U/mg. Human recombinant IFN-α_{2a} was a commercial preparation obtained from Hoffman-La Roche.

IL-1α was diluted to 400 pg/ml blood, IL-1β to 500 pg/ml blood, IL-6 to 200 pg/ml blood, TNF-α to 500 pg/ml blood and IFN-α and IFN-γ to 100 U/ml blood. These dilutions were within the limits of the range of the individual cytokine assays.

Anticoagulants

EDTA (E) (Limclair from Sinclair Pharmaceuticals, UK; a trisodium EDTA preparation in 5 ml ampoules) was used at a dilution of 1.5 mg/ml blood. Aprotinin with EDTA (ET) (Trasylol from Bayer Pharmaceuticals, Germany, a protease inhibitor preparation in 10 ml ampoules containing 200,000 KIU) was used at a dilution of 1000 KIU/ml blood. Heparin (Monoparin from CP Pharmaceuticals, UK, a preparation containing 25,000 IU of sodium heparin in 1 ml ampoules) was used at a dilution of 30 IU/ml blood.

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Cytokine immunoassays

TNF-α, IL-1β, and IFN-γ were measured using IRMA kits from Medgenix (Brussels, Belgium). The TNF-α IRMA range was from 15 to 5000 pg/ml. It was calibrated with the international reference preparation (87/650) (NIBSC), and was used at detection limits of 30 pg/ml plasma. The IL-1β IRMA range was from 50 to 5000 pg/ml. It was calibrated with the international reference preparation (86/680) (NIBSC), and was used at a detection limit of 75 pg/ml plasma. The IFN-γ IRMA range was from 0 to 90 U/ml. It was calibrated with the interim standard preparation (88/606) (NIBSC), and was used at a detection limit of 2.5 U/ml plasma. The IFN-α was measured using RIA kits from Medgenix (Brussels, Belgium). The range was from 0 to 200 IU/ml. It was calibrated with the international reference preparation (82/576) (NIBSC), and was used at a detection limit of 8.0 IU/ml. IL-6 was measured using an RIA method developed by Poole et al. (personal communication),

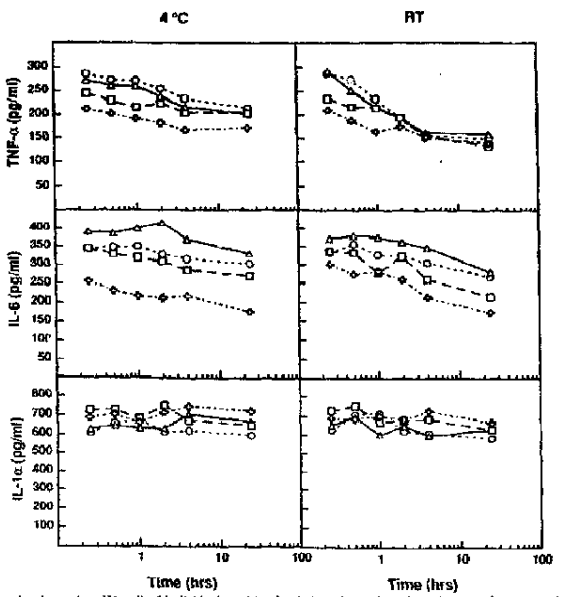

Fig. 1. Concentration (pg/ml or IU/ml) of individual cytokine levels (y axis) against elapsed time before separation of blood (x axis, semi-$\log_{10}$ plot). Samples processed under sterile non-pyrogenic conditions. Each line represents the mean of five volunteer spiked bloods, for each cytokine. Each line represents blood treated as follows: (○) EDTA/Trasylol, (△) EDTA, (□) heparin and (+) serum.

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using the antiserum, G150 BM (NIBSC). The antiserum was used at a final dilution of 1/1,750,000 (equivalent to an initial dilution of 1/350,000). The assay was calibrated with an interim standard preparation (88/514) (NIBSC), and was used at a detection limit of 70 pg/ml/ml. IL-1α was measured using an RIA method developed by Poole et al. (1988) using antiserum S76 BM (NIBSC). The antiserum was used at a final dilution of 1/1,500,000 (equivalent to an initial dilution of 1/300,000). The assay was calibrated with the international reference preparation (86/632) (NIBSC), and was used at a detection limit of 100 pg/ml.

Bloods

Blood was taken from healthy volunteers; administrative, clerical, medical, paramedical and laboratory personnel whose ages ranged from 20 to 55 years. Only volunteers who had not been on any medication and had not donated blood in the preceeding 6 months were used in this study. A total of 63 volunteer bloods were used.

Five volunteer bloods were used per cytokine

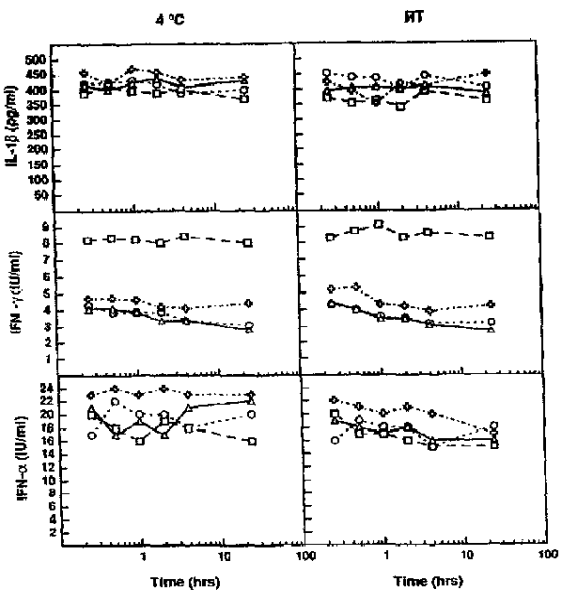

Fig. 2. Concentration (pg/ml or IU/ml) of individual cytokine levels (y axis) against elapsed time before separation of blood (x axis, semi-$\log_{10}$ plot). Samples processed under sterile non-pyrogenic conditions. Each line represents the mean of five volunteer spiked bloods, for each cytokine. Each line represents blood treated as follows: (○) EDTA/Trasylol, (△) EDTA, (□) heparin and (+) serum.

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assay in the first and second studies and three volunteer bloods per cytokine assay for the third study.

Experimental plan

For the first study all blood collection and processing were carried out using sterile non-pyrogenic materials and conditions. 180 ml of venous blood were taken from each volunteer, split into 4 × 40 ml aliquots and each aliquot in turn treated with/without anticoagulants and spiked with a cytokine. These samples were then further split into 20 ml aliquots and stored at 4°C and at RT respectively. At 15 min, 30 min, 1.0 h, 2.0 h, 4.0 h and 24.0 h the blood aliquots were cool spun, separated, flash frozen, and stored at −40°C, prior to assaying. The remaining 20 ml of the venous blood collection were used to ascertain any measurable basal levels of endogenous cytokine.

For the second study all bloods were collected as per normal clinical conditions using commercially available blood collection materials and tubes (Sterilin products, UK). 10 ml potassium EDTA primed plastic tubes, 10 ml lithium heparin primed plastic tubes and 10 ml plain glass tubes were used. No EDTA/Trasylol treated blood was used in this study. 160 ml of venous blood were collected from each volunteer, split into 3 × 40 ml aliquots and each aliquot in turn split into 2 × 20 ml. Only one of the aliquots was spiked with individual cytokines. The other was processed in parallel with/without anticoagulants alone. Each of the 20 ml aliquots were further split into 2 × 10 ml and stored at 4°C and at RT respectively. At 30 min, 2.0 h, and 24.0 h aliquots were cool spun, separated, flash frozen and stored at −40°C prior to assaying. The remaining 20 ml of the venous blood collection were used to ascertain any measurable basal levels of endogenous cytokine.

In the third study, blood from three volunteers was collected per cytokine assay and processed under sterile non-pyrogenic conditions, as described above for the first study. These samples were then cool spun, separated and flash frozen within 30 min of collection. Aliquots of these plasma/serum samples were subjected to repeated cycles of freeze thawing at 4°C, and at RT.

Results

The data generated were expressed graphically in terms of an absolute concentration as a measure of recovery of the spiked cytokines against timed separation, or the number of times the samples were freeze thawed (see Figs. 1, 2, 3 and 4). Basal levels of cytokine were measured in all volunteer blood samples used. In the second study, samples collected under normal clinical conditions had parallel 'no spike, buffer treated samples' processed alongside to establish any overt induction of cytokine production.

The data generated were also analysed in terms of % change from the first sample separated at 15 min. for samples generated in the first study under non-pyrogenic, sterile conditions; % change from first sample separated at 30 min, for samples generated in the second study using normal blood collection materials and procedures; and% change from single freeze thawed plasma/serum samples generated in the third study.

The results expressed as % change from initial value, and absolute concentration as a measure of recovery, were compared using analysis of variance which controls for subject (volunteer) and time when looking for a tube effect, and subject and tube (anticoagulant) when looking for a time effect. Because multiple comparisons were made, the significance level for p was taken as 0.01.

TNF-α

When blood was collected under sterile pyrogen-free conditions there was significant reduction in measurable TNF with time, controlling for subject and anticoagulant, at both at 4°C and RT ($p < 0.001$ at 4°C, and $p < 0.001$ at RT), as shown in Fig. 1. Although stability expressed as % change was similar for the anticoagulated and serum tubes there was a significant difference between these tubes in terms of absolute TNF-α recovery ($p < 0.001$ at 4°C, and $p < 0.001$ at RT) with serum tubes consistently giving lower recovery of TNF than heparin, EDTA or EDTA/Trasylol treated bloods. There was no advantage in adding the aprotinin (Trasylol) to EDTA bloods. The results using commercially available tubes were almost identical, again with a significant reduction in measurable TNF with time at both 4°C ($p < 0.001$) and at RT ($p < 0.001$), the loss of

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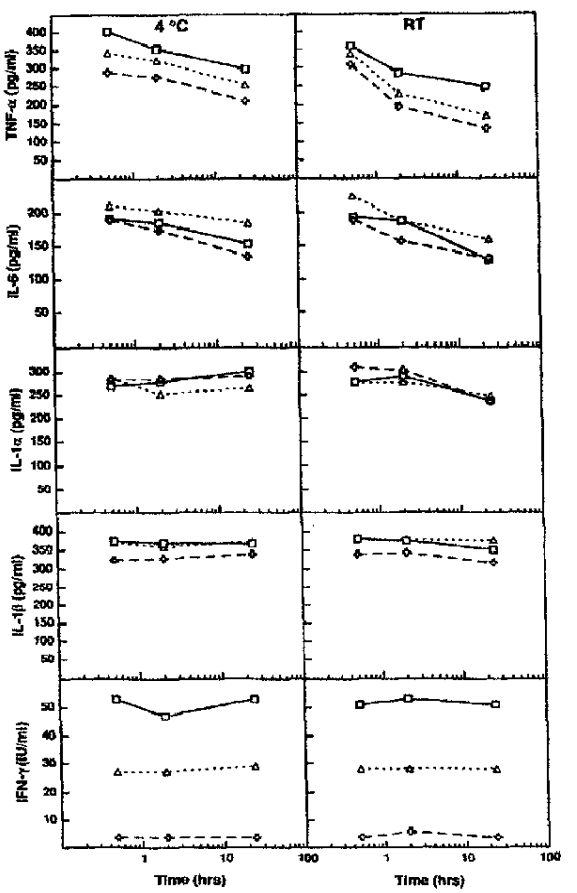

Fig. 3. Concentration (pg/ml or IU/ml) of individual cytokine levels (*y* axis) against elapsed time before separation of blood (*x* axis, semi-$\log_{10}$ plot). Samples processed using normal blood collection methods. Each line represents the mean of five volunteer spiked bloods, for each cytokine. Each line represents blood treated as follows: (▲) EDTA, (□) heparin and (+) serum.

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stability being approximately doubled at RT compared to 4°C, as shown in Fig. 3. There was no effect on TNF levels after six repeat freeze/thaw cycles although again there was a significant tube effect ($p < 0.001$) with serum samples consistently giving lower recovery than anticoagulated samples, as shown in Fig. 4.

IL-6

With bloods collected under sterile pyrogen-free conditions there was a significant reduction in IL-6 stability with time at both 4°C ($p < 0.001$) and RT ($p < 0.001$), although the change was less pronounced at 4°C (Fig. 1). There was also a significant difference between tubes in terms of recovery ($p < 0.001$), with EDTA treated bloods giving a higher recovery of the spiked cytokine at both 4°C and at RT. The recovery from serum tubes was consistently low. With bloods collected in commercially available tubes there was a significant reduction in stability and recovery with time at both 4°C ($p < 0.001$ for stability and $p = 0.014$ for recovery) and at RT ($p < 0.001$ for stability, $p < 0.001$ for recovery) as shown in Fig. 3. Again EDTA treated bloods gave a higher recovery of the spiked cytokine at both 4°C and at RT. The recovery from serum tubes was low. There was no significant freeze/thaw effect up to three cycles for EDTA, EDTA/Trasylol and serum. However heparinized plasma showed inconsistent stability and recovery as shown in Fig. 4.

IL-1α

For blood processed under sterile pyrogen-free conditions there was no significant time effect for tubes stored at 4°C ($p = 0.280$) but there was a significant time effect in IL-1α levels in tubes stored at RT ($p < 0.001$) as shown in Fig. 1. This was also true of bloods collected in commercial tubes, with IL-1α appearing stable at 4°C ($p = 0.655$), but not when stored at RT ($p < 0.001$) as shown in Fig. 3. There was no significant change in the stability of IL-1α levels in plasma/serum samples freeze thawed 6 times. However the recovered concentrations of IL-1α varied, with heparinized plasma giving a significantly higher recovery ($p < 0.001$) at RT as shown in Fig. 4.

IL-1β

For blood processed under sterile pyrogen-free conditions there was no significant time effect at either 4°C or RT, and no difference between the four tube types as shown in Fig. 2. This was also true of bloods collected in commercial tubes, as shown in Fig. 3. However the concentration of *IL-1β recovered in serum samples* in this study was lower ($p = 0.001$ at 4°C, $p < 0.001$ at RT). There was no significant effect of up to six freeze thaw cycles as shown in Fig. 4.

IFN-γ

With bloods collected under sterile pyrogen-free conditions there was a significant reduction in IFN-γ levels with time at both 4°C ($p < 0.001$) and RT ($p < 0.001$), in ET, E and S tubes ($p < 0.001$), as shown in Fig. 2 but there was no similiar pattern of degradation in the H tubes. Furthermore, although all samples were spiked with the same amount of IFN-γ, the recovered concentration from heparinized plasma was almost twice as much as for EDTA treated bloods as shown in Fig. 2. With normal blood collection procedures there was no significant time effect with any of the tubes, either at 4°C or RT as shown in Fig. 3. However, the recovered concentration in EDTA treated bloods was five times that of serum samples, and recovery from heparinized bloods was ten times that of serum samples as shown in Fig. 3. This was the only cytokine studied for which there was a difference in stability between the sterile/non-pyrogenic tubes and the commercial tubes. In view of the observations made for this cytokine, a further three heparinized blood samples were processed with and without spiked IFN-γ under sterile pyrogen-free conditions and subjected to timed separation. There was no evidence of induced IFN-γ in unspiked heparinized plasma. IFN-γ was stable for up to six freeze thaw cycles, as shown in Fig. 4.

IFN-α

There was no significant time effect for samples stored at 4°C but there was a significant time effect in IFN-α levels in tubes stored at RT ($p < 0.001$) as shown in Fig. 2. ET treated bloods from four of five volunteers consistently gave recoveries of around 120% when compared to the

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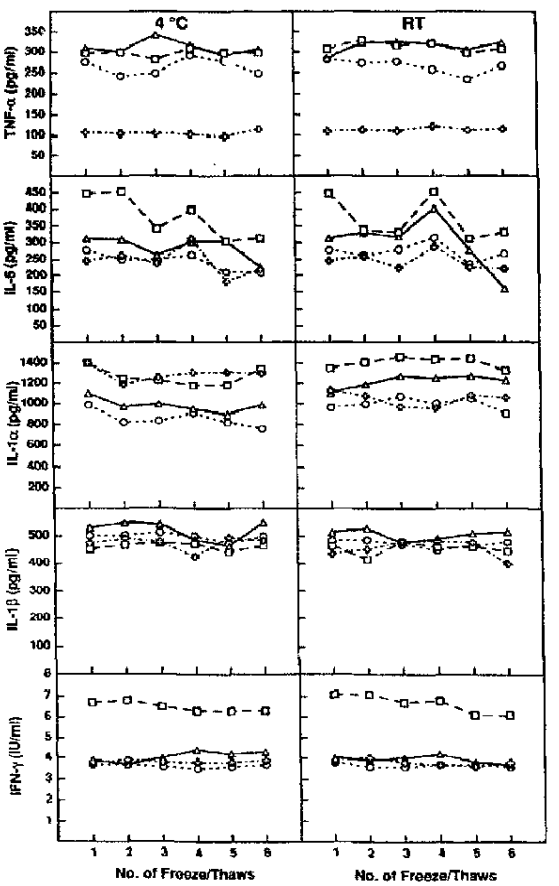

Fig. 4. Concentration (pg/ml or IU/ml) of individual cytokine levels (y axis) against number of freeze thaws (x axis). Samples processed under sterile non-pyrogenic conditions and separated from cellular components within 30 min of collection. Each line represents the mean of three volunteer spiked bloods, for each cytokine. Each line represents blood treated as follows: (○) EDTA/Trasylol, (△) EDTA, (□) heparin and (+) serum.

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first sample ($p < 0.001$). Serum samples consistently gave higher levels of recovery of IFN-α. IFN-α was only assessed in the first study.

Basal levels and induced cytokines in volunteer blood samples

63 volunteer blood samples were used in this study. Each donation in turn generated three plasmas and one serum sample for evaluating basal levels Amongst the volunteer population the median value for endogenous IL-6 was 24 pg/ml (0–33 pg/ml). These values were below the reliable detection limits of the assay. Likewise the median value for TNF-α levels amongst the volunteer population was 2.0 pg/ml (0–4.0 pg/ml), again below the reliable detection limits. There were no measurable levels of IL-1α, IL-1β, IFN-α and IFN-γ amongst the volunteer population. There was no evidence of cytokine induction in unspiked volunteer bloods, collected and processed alongside spiked bloods of the same volunteers, when using normal blood collection tubes and materials.

Discussion

The aim of conducting these experiments with bloods spiked with human recombinant cytokines was to simulate conditions that are likely to affect the reliable immunoassay measurement of cytokines in blood samples. In this respect there are drawbacks in that the genetically engineered and stabilized cytokines may not behave in a similar manner to the naturally occurring product and that their pattern of degradation may not be the same. Moreover, ex vivo addition of cytokines to blood will probably not give an exact reflection of levels after in vivo administration, since soluble receptors/binding proteins may be induced after in vivo administration of recombinant cytokine (Lantz et al., 1990) and these may alter assay results.

However, these assays have since been put to use in clinical settings and have generated useful information. To illustrate an example: a patient with metastatic disease was entered into a phase II study to receive IL-2 by infusion in conjuction with chemotherapy. It soon became evident that the bioavailability of the IL-2 in the absence of carrier protein was significantly reduced. When this was corrected the patient showed all symptoms of IL-2 dose related toxicity. The serum TNF-α levels correlated closely with this toxicity (Miles et al., 1991).

There have been claims that the use of aprotinin may improve the conservation of cytokines in blood (Cannon et al.,1988; Endres et al., 1988). However in this study there was no advantage to adding aprotinin to EDTA tubes in terms of improved stability. The possibility of ex vivo induction of cytokines by the presence of very low levels of endotoxin in sterilized, but not pyrogen-free, material used for normal blood collection was also investigated. The Sterilin blood collection tubes and Sabre syringes used in experiments simulating normal blood collection, were not found to contribute to any ex vivo induction of cytokines. However, there have been reports by Freeman et al. (1990) and McLaughlin et al. (1990) of ex vivo induction of cytokines by high levels of endotoxin in lithium heparin blood collection tubes. Riches et al. (1990) showed that bloods collected in heparinized Vaccutainer tubes did show ex vivo induction of IL-6. It should also be noted that heparinized plasma samples are generally more difficult to handle after the freezing/thawing procedure, because of protein precipitation and the need to respin thawed heparinized plasma prior to assay. Others (e.g., Exley and Cohen, 1990) have studied the stability of TNF-α in blood and reported similar results to ours.

In conclusion the stability of the spiked recombinant cytokines in whole blood was variable but in general appeared to be improved by storage of blood samples at 4°C and/or rapid separation. Samples treated with EDTA performed most consistently with least assay problems. As to other suitable anticoagulants, Engelberts et al. (1991) have found TNF-α levels in blood collections were better preserved using oxalate/sodium fluoride primed collection tubes. In view of the many kit immunoassays and laboratories measuring cytokines in blood, there is a need to work towards a standardization of conditions and methods to extract reliable and consistent information. It is hoped that this study will contribute to this process.

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